Polycationic star polymers with hyperbranched cores for gene delivery

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Abstract

Core-shell type stars synthesized via atom transfer radical polymerization were used for the delivery of nucleic acids. The interior of the stars consisted of hyperbranched poly(arylene oxindole), while the arms were composed of poly(N,N-dimethylaminoethyl methacrylate). The length of the star arms varied in degree of polymerization (DP) from 14 to 98. The hydrodynamic radius of the structures measured in water indicated the presence of small aggregates, while isolated stars ranging in size from 14 to 29 nm were seen in organic solvent. The phase transition temperatures of the stars in water, measured in basic conditions, were shifted to lower values with increasing DP of the arms. Stable polyplexes of stars with plasmid DNA were formed. Their size varied from 300 nm to 400 nm, depending upon the DP of arms. The zeta potential of the polyplexes was positive, which facilitated their cellular uptake. The DP of the arms influenced the transfection efficiency of HT-1080 cells, demonstrating that stars are promising candidates for synthetic gene vectors.

Keywords: polyplexes, star polymers, gene therapy

1. INTRODUCTION

The design of effective systems for the protection of genetic material against degradation and for transferring nucleic acids to a specific target is an important task in gene transfection studies. The use of viruses as carriers has yielded promising results, but the side effects caused by immunogenic reactions highlight the need for constructing safe and less pathogenic synthetic substitutes. An approach to meet these requirements that has been developed in the last decade is the use of polycationic polymers [1]. The amino groups of these polymers are able to condense DNA or RNA into so called polyplexes. Recent progress in star polymer synthesis and characterization [2, 3] has opened the route for creating star polymer-based vectors for gene therapy [4, 5].

Polycationic star polymers used for the formation of polyplexes are mainly made of polyethyleneimine [6], poly(N,N-dimethylaminoethyl methacrylate) (PDMAEMA) [7-9] and poly(N,N-dimethylaminopropylacrylamide) [10]. The most popular are PDMAEMA stars. In most cases these are synthesized using "arm first" or "core-first" methods.

PDMAEMA stars have been obtained via an "arm-first" method by group transfer polymerization, in which ethylene glycol dimethacrylate or bis(methacryloyloxyethyl) methylamine was used as the crosslinking agent to interconnect individual PDMAEMA chains at one of their ends [7, 8]. In "the core first" approach, multifunctional cores have been used as macroinitiators for the polymerization of the arms. Typically, atom transfer radical polymerization (ATRP) has been used to create arms of DMAEMA, while several multifunctional macroinitiators possessing bromo functionalities have served as the star cores. Low molar mass compounds, such as pentaerythritol [11] and its derivative [12], tris(α-bromoisobutyryl) N-methyl triethanolamine [13] and various calixarene derivatives [14, 15] or functionalized cyclic saccharides [16-18], have been used. Tam et al. grafted DMAEMA onto four arm poly(ethylene oxide) [19, 20] or onto C_{60}-fullerene [21]. Different inorganic cores have also been applied to obtain stable star nanoparticles for gene therapy, including cores made of silica [22], silsesquioxanes [18, 23] and γ-Fe_2O_3 [24].

There are only a few examples using relatively large, hyperbranched core molecules for the preparation of PDMAEMA stars. Hyperbranched poly(3-ethyl-3-(hydroxymethyl)oxetane (HBPO) functionalized with bromoester or trithiocarbonate moieties has been used to synthesize PDMAEMA stars by ATRP [25-27] and reversible addition fragmentation polymerization [28]. In addition, Yan et al. [29] studied the anionic polymerization of DMAEMA using hydroxyl terminated HBPO, reacted with potassium hydride, as the macroinitiator. Other examples of such cores are commercially available hyperbranched polyesters, which have been used to synthesize stars with block copolymer arms of poly(lactide)-*b*-PDMAEMA [30], and alanine based hyperbranched poly(ester amide) with thioether functionalities, which has been used to create stars with PDMAEMA arms [31].

Positively charged polymer materials, both linear and branched, are able to condense nucleic acids into polyplex structures. The condensation of DNA is more efficient for PDMAEMA star polymers in comparison to linear analogues [9, 16]. The dense molecular star structure and the higher number of functional groups for a given degree of polymerization compared to linear polymers used in polyplexes have improved the effectiveness of stars for DNA

complexation [7, 8]. Because of their small sizes, stars can form smaller polyplexes, which are believed to be more desirable for achieving high transfection efficiency [9, 32].

Despite several reports concerning the synthesis and application of DMAEMA stars for gene delivery, many questions remain to be answered, especially considering that contradictory data and conclusions have been reported. Based on verified experimental data, the molar mass, the functionality of stars, their conformation in water and culture media solution, and the charge density are expected to exert significant influences on transfection efficiency and should be examined very carefully. Even the molar masses of the stars, which are basic characteristics, are frequently only estimated based upon GPC calibrated with linear polymers [7, 9, 16, 32]. Thus, the absolute molar mass and dispersity data are hardly available. The number and lengths of star arms are expected to be essential for transfection. However, their influence remains a subject of discussion, as transfection efficiency has been reported to both decrease [7] and increase with increasing arm length [16].

It is known that transfection efficiency increases with the polymer concentration, but this increase is accompanied by a decrease in cell viability [7, 8, 16]. In general, cytotoxicity increases with the increasing molar mass of the polycationic polymer. At lower ratios of the number of amino groups in the star vector to the phosphate groups of the nucleic acid backbone (N/P), nucleic acids cannot be efficiently condensed and the polyplex cannot enter the cell. With increasing N/P, transfection efficiency increases but cell viability decreases [7, 9, 16, 20, 32].

Investigations on the influence of all the discussed parameters on the properties of polyplexes based upon PDMAEMA stars are needed.

In this paper, we report novel well-defined star polymer delivery systems that are based on star polymers with a hydrophobic hyperbranched poly(arylene oxindole) core and cationic PDMAEMA arms. Hyperbranched poly(arylene oxindole core) was applied for the synthesis of star polymers because of its high degree of branching and a large number of reactive functional groups, which led to multiarm structures. The introduction of such hydrophobic polymer into the DNA carrier structure was expected to improve its cellular uptake through the lipophilic cell membrane and, as the result, increase the transfection efficiency, as it was studied earlier [16, 33].

To the best of our knowledge stars with hyperbranched cores have never been used in gene transfection experiments. Complexation of the obtained polymers with pDNA using various

N/P ratios was studied to provide information about the efficiency of polyplex formation, polyplex size, and, as a result, transfection efficiency. In addition, to obtain a safe and efficient gene delivery system, the limit of the molar mass of the carrier was examined to avoid an undesired increase in toxicity.

2. EXPERIMENTAL

2.1. Materials

1,2-Dichlorobenzene (99%), 1,1,4,7,10,10-hexamethyltriethylenetetraamine (HMTETA, 97%), copper (I) bromide (CuBr, 99.999%), copper (II) bromide ($CuBr_2$, 99%), p-xylene (99%), Dulbecco's Modified Eagle's Medium (DMEM) and kanamycin sulfate were purchased from Sigma Aldrich. Lysogeny Broth (LB) medium for cultivation of the bacteria was purchased from QBIOgene (USA). Branched polyethylenimine (PEI) of M_n=25 000 g/mol and ethidium bromide solution 10 mg/mL in H_2O was purchased from Sigma Aldrich and used as received. N,N-dimethylaminoethyl methacrylate (DMAEMA, 98%) was purchased from Sigma Aldrich and purified by distillation prior to use. DOWEX MARATHON MSC ion exchange resin (Aldrich) was transformed into the H^+ type using 1.6 M HNO_3. Methanol (99.8%) was purchased from POCh and used as received. Acetone (99.5%) and ethanol (96%) were purchased from POCh and filtered through membrane filters with a pore size 0.02 μm prior to use.

Fetal bovine serum (FBS), L- glutamine, penicillin, streptomycin, amphotericin B, trypsin (0.25% in 1 mM EDTA) and phosphate buffered saline (PBS) were purchased from PAA Laboratories GmbH and used for the cell culture experiments. AlamarBlue Cell Viability Reagent was purchased from Invitrogen.

Plasmid DNA (pDNA) pMetLuc2-control vector with 4784bp purchased from Clontech was prepared using the Plasmid DNA Maxi Kit (Omega Bio-Tek, USA).

2.2. Synthesis of poly(arylene oxindole) core

The synthesis and characterization of the poly(arylene oxindole) (PArOx) core was described in our previous paper [34]. The PArOx with 28 bromoester groups was used as macroinitiator of the atom transfer polymerization of DMAEMA. The molar mass of PArOx was M_n=21 000 g/mol and M_w/M_n=2.17.

2.3. Synthesis of star polymers with poly(N,N-dimethylaminoethyl methacrylate) arms

PArOx (20×10^{-3} g, 9.5×10^{-7} mol), CuBr (3.8×10^{-3} g, 2.65×10^{-5} mol), $CuBr_2$ (3×10^{-4} g, 1.34×10^{-6} mol) and HMTETA (6.18×10^{-3} g, 2.68×10^{-5} mol, 7.3×10^{-3} mL) ([CuBr]:[$CuBr_2$]:[HMTETA] in the ratio of 1:0.05:1) were dissolved in 1 mL of 1,2-dichlorobenzene in a Schlenk flask under nitrogen with a magnetic stirrer and degassed twice using freeze-vacuum-thaw cycles. Next, N,N-dimethylaminoethyl methacrylate (DMAEMA

0.42 g, 2.67 × 10^{-3} mol, 0.45 mL) (monomer to 1,2-dichlorobenzene ~1:2 v/v) was added and the solution was degassed again. The flask was placed in an oil bath and heated at 45 °C. Monomer conversion was measured by gas chromatography using p-xylene as an internal standard. Samples were taken during the polymerization and analyzed without purification, using gas chromatography to check the monomer conversion and GPC to determine the molar mass and dispersity. After the desired conversion was obtained, THF (5 mL) was added, and the solution was passed through a column with DOWEX-MSC-1 ion exchange resin to remove the copper. The solution without copper was dialyzed, first against methanol and then against water (SpectraPor membrane with MWCO 1000 g/mol), and dried by lyophilization to prevent polymer aggregation.

2.4. Selective alkaline hydrolysis of ester bonds between the arms and the core of the stars

Star polymer (0.1 g) was dissolved in THF (10 mL) in a round bottom flask fitted with a condenser and a nitrogen inlet. A KOH solution (2 mL, 1 M in ethanol) was added to the flask, and the reaction mixture was refluxed at 60 °C for 15 min. After this time, the next portion of KOH solution (2 mL, 1 M in ethanol) was added to the flask, and the reaction was again refluxed at 60 °C for 15 min. The solution was dialyzed (SpectraPor membrane with MWCO 1000 g/mol), first against methanol and then against water. The water solution was neutralized using 1 M HCl and dialyzed again. The solvent was removed by lyophilization.

2.5. Measurements

NMR spectra were recorded using a Bruker Ultrashield 600 spectrometer (600 MHz for ^{1}H). The resonances are given in ppm, referenced to the tetramethylsilane (TMS) peak.

The molar masses and molar mass distributions of the star polymers were determined using gel permeation chromatography (GPC-MALLS) with a differential refractive index detector (Δn-2010 RI WGE Dr. Bures) and a multiangle laser light scattering detector (DAWN EOS from Wyatt Technologies). GPC was performed in DMF at 45°C with a nominal flow rate of 1 mL/min using set of columns: 100 Å (Polymer Standard Service) + 2 x PL Gel Mixed-C (Polymer Laboratories). The results were evaluated using the ASTRA 5 software from Wyatt Technologies.

The refractive index increments (*dn/dc*) of the stars were calculated from Eq. (1):

$$\frac{dn}{dc} = w_{PArOx}\left(\frac{dn}{dc}\right)_{PArOx} + w_{DMAEMA}\left(\frac{dn}{dc}\right)_{PDMAEMA} \quad (1)$$

where w_{PArOx} is the weight ratio of the core and w_{DMAEMA} is the weight ratio of DMAEMA in the star polymer, $\left(\frac{dn}{dc}\right)_{PArOx}$ is the refractive index increment of the PArOx core and $\left(\frac{dn}{dc}\right)_{PDMAEMA}$ is the refractive index increment of the PDMAEMA.

The refractive index increments of PArOx (*dn/dc*=0.149 mL/g) and linear PDMAEMA (*dn/dc*=0.056 mL/g) were independently measured in DMF using a SEC-3010 dn/dc WGE Dr. Bures differential refractive index detector. These values were used for calculating the refractive index increment of the stars S1-S8 (Table 1) according to Eq. (1).

Dynamic light scattering (DLS) measurements were performed for the star polymers and polyplexes on a Brookhaven BI-200 goniometer with vertically polarized incident light of wavelength λ=632.8 nm, supplied by a He-Ne laser operating at 35 mW, and a Brookhaven BI-9000 AT digital autocorrelator. The autocorrelation functions were analyzed using the constrained regularized algorithm CONTIN. The measurements were made at a 90° angle. The dispersity of particle sizes was given as $\frac{\mu_2}{\overline{\Gamma}^2}$, where $\overline{\Gamma}$ is the average value of relaxation rates Γ and μ_2 is its second moment. These values were obtained from cumulant analysis.

Before DLS analysis, the star polymer solutions were filtered through membrane filters with nominal pore sizes of 0.2 μm (ANATOP 25 PLUS, Whatman). The solvents (except culture medium DMEM) used to prepare the solutions for DLS were first filtered through a 0.2 μm nitrocellulose filter (Millipore) and then through a 0.02 μm membrane filter (ANATOP 25 PLUS, Whatman) in a laminar flow cabinet prior to use.

The cloud point temperature (T_{cp}) of the star polymers was determined on a Jasco V530 UV-VIS spectrophotometer with a cuvette thermostated by a Medson MTC-P1 Peltier thermocontroller. The SPECTRA MANAGER program enabled advanced analysis of the obtained spectra. The transmittance of the aqueous star solutions (c=5 g/L) was measured at λ=700 nm. The cloud point values were determined as the temperature at which the transmittance of the polymer solution reached 50% of its initial value.

Zeta potential measurements were performed on a Zetasizer Nano ZS 90 (Malvern Instruments) in disposable folded capillary cells in triplicate.

Cryogenic Transmission Electron Microscopy images were obtained using a Tecnai F20 TWIN microscope (FEI Company, USA) equipped with a field emission gun, operating at an

acceleration voltage of 200 kV. Images were recorded on an Eagle 4k HS camera (FEI Company, USA) and processed with TIA software (FEI Company, USA). Specimens were prepared by vitrification of aqueous polymer solutions on grids with holey carbon film (Quantifoil R 2/2; Quantifoil Micro Tools GmbH, Germany). Prior to use, the grids were activated for 15 seconds in oxygen/argon plasma using a Fischione 1020 plasma cleaner (E.A. Fischione Instruments, Inc., USA). Samples were prepared by applying a droplet (2.1 µL) of solution to the grid, blotting with filter paper and immediately freezing in liquid ethane using a fully automated Vitrobot Mark IV (FEI Company, USA) blotting device. After preparation, the vitrified specimens were kept under liquid nitrogen until they were inserted into a Gatan 626 cryo-TEM-holder (Gatan Inc., USA) and analyzed in the TEM at -178 °C.

2.6 Plasmid DNA preparation

The plasmid pMetLuc2-control vector (Clontech) was amplified in Escherichia coli, DH5 alpha strain, cultured in LB Broth (MILLER) medium (QBIOgene, USA) with 50 µg/mL of kanamycin. The plasmid DNA was purified using the Plasmid DNA Maxi Kit (Omega Bio-Tek, USA) according to manufacturer's protocol. The DNA concentration was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc).

2.7 Formation of complexes of star polymers with DNA

A suitable amount of star polymer solution in DMEM (c=0.5 mg/mL) was added to a fixed volume of plasmid DNA solution in DMEM (300 µL, c=0.1 mg/mL) to achieve the desired nitrogen/phosphate (N/P) ratios. The obtained solutions were mixed and incubated at room temperature for 30 min. Polyplexes prepared by this method were used for the DLS and zeta potential measurements.

2.8 Agarose Gel Electrophoresis

For the agarose gel electrophoresis experiments, polyplexes were prepared with 330 ng of plasmid DNA in a total volume of 20 µl DMEM medium, which was free of FBS and supplements. To obtain different N/P ratios, an appropriate amount of polymer was added to the DNA solution, vortexed and incubated for 30 min at RT to allow polyplex formation. Afterwards 2 µL of loading buffer was added and the entire reaction volume was loaded onto 1.5% agarose gel containing 1 µg/mL ethidium bromide. Electrophoresis was performed at a voltage of 150 V for 10 min in TAE (Tris-acetate EDTA) running buffer. The gel was analyzed on a UV illuminator (Syngene) to visualize the location of the polyplexes compared to naked plasmid DNA.

2.9 Cell culture

HT-1080 human fibrosarcoma cells (ATCC#CCL-121) were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine, penicillin, streptomycin and amphotericin B at 37 °C and 5% CO_2. After reaching 90% confluency, the cells were divided and passaged to three 175 cm^3 bottles.

2.10 Cytotoxicity of star polymers

HT-1080 cells were seeded in DMEM growth medium in 24-well plates at the density of 8 x 10^4 cells per well 24 h prior to the cytotoxicity experiments. The next day, the medium was discarded and replaced with 500 µL of fresh complete culture medium with various concentrations of polymer. The plates were incubated for an additional 24 h, then the medium was removed and the plates were rinsed with warm PBS buffer. After adding 400 µL of 10% AlamarBlue reagent in complete growth medium to each well, the plates were incubated for 1 h. Then, the fluorescence at 560 nm excitation wavelength and a 590 nm emission wavelength was monitored using a VICTOR^{TM} Multilabel Plate Reader (Perkin Elmer, USA). The viability (in %) was calculated by comparing treated cells with control cells that were not treated with the polymer, which represented 100% viability. The tests were performed four times.

2.11. *Cytotoxicity of polyplexes and transfection efficiency studies*

The polyplex cytotoxicity assay was performed similarly to that described for the star polymers. The polyplexes were prepared by combining 1 µg of DNA (in a total volume of 100 µL of supplement-free DMEM) with an appropriate amount of the star polymer, corresponding to the different N/P ratios, vortexing and then incubating for 30 min at room temperature.

Transfection experiments were performed on HT-1080 cells using the plasmid pMetLuc2-control vector. The HT-1080 cells were seeded in DMEM complete growth medium in 12-well plates at the density of 16 x 10^5 cells per well 24 h prior to the transfection experiments. Shortly before transfection, the medium was discarded and replaced with 1 mL of fresh, warmed, supplemented DMEM medium. Preformed polyplex solutions were added to the cells in the culture medium. The plates were incubated for an additional 48 h at 37 ˚C. After this time, the medium was aspirated, the cells were washed with warm PBS buffer, 0.5 mL of fresh medium was added and the cells were incubated for 8 h. Then, 50 µL of culture medium was taken for secreted luciferase activity detection. Metrida secreted luciferase activity in the

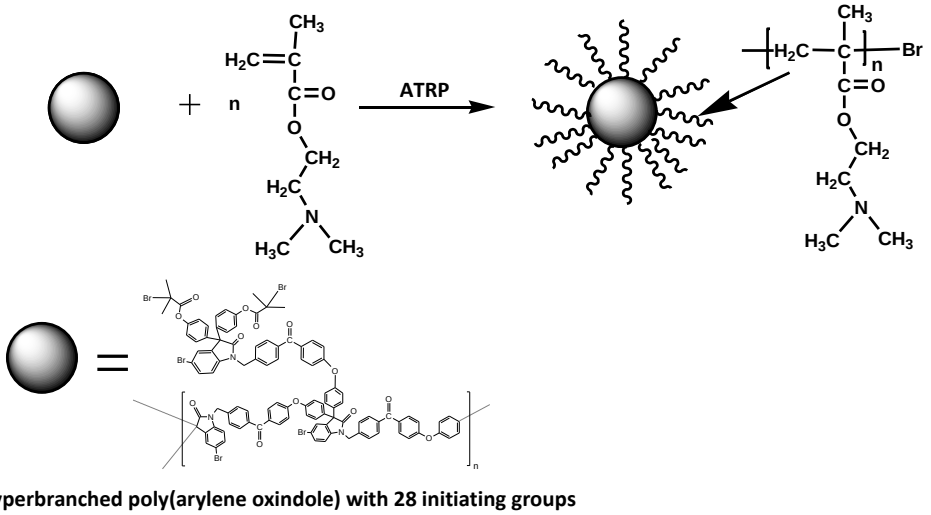
culture medium was measured. The transfection efficiency was evaluated indirectly based on the average of three luminescence measurements performed in 96-well plates with a 1 s exposition time.

3. RESULTS AND DISCUSSION

3.1. Synthesis of the star polymers with DMAEMA arms

Star polymers of N,N-dimethylaminoethyl methacrylate were obtained via atom transfer radical polymerization (ATRP) (Scheme 1). Hyperbranched poly(arylene oxindole) (PArOx) with a degree of branching equal to 100% and 2-bromoester groups, which initiated the atom transfer polymerization of DMAEMA, was used as the core. The number of initiating groups was calculated to be 28 using the Frey equation [35], which relates the number of dendritic and terminal units to the degree of polymerization. The core polymer was obtained, as previously described [34], via polycondensation of the 5-bromo-1-[4-(4-phenoxybenzoyl)benzyl]-isatin (AB_2 monomer). Subsequently, the functional groups were modified to 2-bromoester groups to introduce the initiating functions. The absolute molar mass of the core measured by GPC-MALLS was M_n=21 000 g/mol and M_w/M_n = 2.17.

The polymerization process was investigated using conditions similar to those used by the Matyjaszewski group to obtain linear PDMAEMA with a narrow molar mass distribution [36]. The initiator to monomer molar ratio was 1:2800, which corresponds to 100 moles of DMAEMA per one mole of initiating sites, assuming that the core had 28 2-bromoester groups. Copper bromide (I), copper bromide (II) and N,N,N’,N”,N”’,N”’-hexamethyltriethylenetetraamine ligand ([CuBr]:[$CuBr_2$]:[HMTETA] in the ratio of 1:0.05:1) was used as a catalytic system in 1,2-dichlorobenzene (2:1 v/v to monomer) at 45°C.



Scheme 1. Synthesis of the PDMAEMA stars.

A series of star polymers with molar masses M_n ranging from 81 000 to 370 000 g/mol were obtained (Table 1). The molar masses and molar mass dispersities of the stars were measured by gel permeation chromatography with multiangle laser light scattering detection (GPC-MALLS) in DMF with 5 mM LiBr. The molar mass distributions obtained for all the stars were relatively narrow and monomodal (Fig. 1). The polymerization process was carried out to low monomer conversion to prevent star-star coupling reactions. As expected, the hydrodynamic volumes of the stars increased with the growth of the arm length.

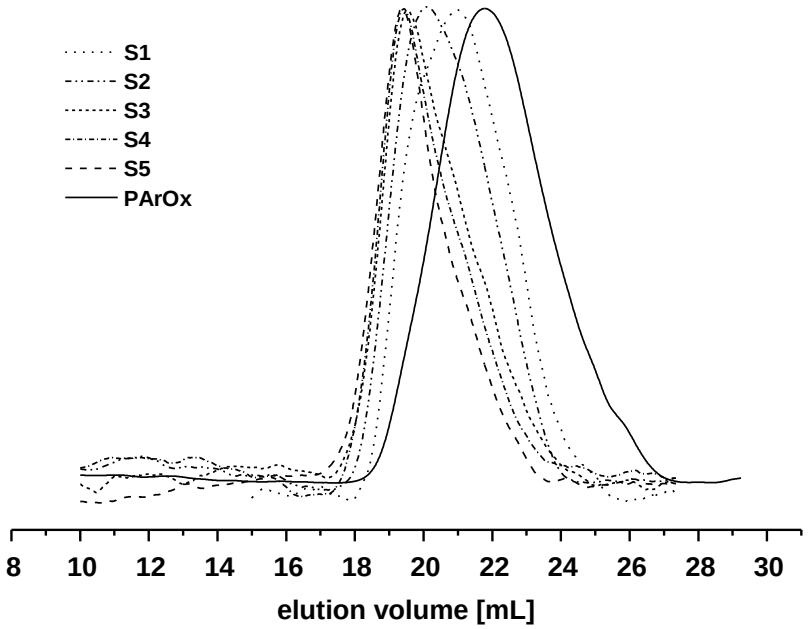


Fig. 1. Chromatograms (RI traces) of the PDMAEMA stars (DMF, 1 mL/min).

Table 1. Properties of the PDMAEMA stars obtained via ATRP.

Sample	DP_{arm} [a]	$M_{n\ GPC\text{-}MALLS}$ [b] [g/mol]	M_w/M_n	dn/dc [c] [mL/g]
S1	14	81 500	1.49	0.090
S2	18	100 000	1.69	0.084
S3	22	117 600	1.58	0.077
S4	25	131 200	1.74	0.073
S5	34	168 800	1.79	0.070
S6	44	213 000	1.64	0.067
S7	79	370 000	1.54	0.065
S8	98	450 000	1.84	0.063

[a]calculated from M_n measured by GPC-MALLS
[b]measured by GPC-MALLS in DMF
[c]calculated from Eq. (1)

Confirmation of the star functionality was achieved by alkaline hydrolysis of the stars S2, S7 and S8 (Table 2). The hydrolysis conditions were chosen to cleave only the ester linkages between the core and the arms and to preserve the polymethacrylic backbone. This type of reaction has been successfully used previously for different types of star polymers [37-42]. The data confirmed complete hydrolysis and were consistent with the molar masses of the stars measured by GPC-MALLS. Similar results were obtained in our earlier reports for other star polymers with polymethacrylate arms and poly(arylene oxindole) cores [34, 41, 42].

Hydrolyzed arms were purified by dialysis, first against methanol and then against water, using membrane with a MWCO 1000 g/mol, dried by lyophilization and characterized by GPC-MALLS (Table 2, Figure S1, Supporting Information). The refractive index increment used to determine the M_n of the PDMAEMA arms was established in independent measurements and was equal to 0.056 mL/g.

The real number of the star arms was subsequently calculated from Eq. (2):

$$f_{calcd} = (M_{n\ star} - M_{n\ core})/M_{n\ arm} \quad (2)$$

Table 2. The experimental functionality, molar masses and dispersities before and after alkaline hydrolysis of PDMAEMA stars.

Sample	$M_{n\ of\ star}$ [a] [g/mol]	$M_w/M_{n\ of\ star}$	$M_{n\ of\ arms}$ [a] [g/mol]	$M_w/M_{n\ of\ arms}$ [a]	f_{calc} [b]
S2	100 000	1.69	3 100	1.27	25.5
S7	370 000	1.54	12 500	1.11	28
S8	450 000	1.84	15 700	1.04	27.3

[a] measured by GPC-MALLS in DMF
[b] calculated from Eq. (2)

The number of initiating groups, calculated according to the Frey equation [35], in the hyperbranched macroinitiator was equal to 28. The calculated average values for the number of arms in the stars *f* (Table 2) were very close to theoretical values, suggesting that almost all the bromide groups of poly(arylene oxindole) initiated DMAEMA polymerization.

The molar mass distribution of the arms was relatively narrow, suggesting that the stars had arms of uniform length. The values obtained were also much lower (Table 2) than the dispersity of the stars.

The structural elements of the stars can be observed in their NMR spectra. The measurements were performed in two solvents with different affinities for the components of the stars. The ^{1}H NMR spectra in chloroform (Fig. 2), which is a good solvent for both the core and the arms, displayed peaks for α-methyl groups and methylene groups in the methacrylate backbone at δ =0.8-1.1 ppm (a) and 1.7-2.0 ppm (b), respectively. Proton signals from the methylene groups in pendant chains were found at δ = 2.5-2.6 ppm (d) and δ = 3.9-4.1 ppm (e), and signals from the methyl protons of the amino group were at δ = 2.1-2.4 ppm (c). Signals corresponding to the aromatic protons of the core were observed in the range of 7.0-7.8 ppm.

The spectra obtained in deuterated water, which is only a good solvent for the hydrophilic arms of the star, had broader signals because of the lower mobility of the chains. Only the signals corresponding to groups present in the arms were visible. The aromatic protons of the hyperbranched core could not be observed in the spectra registered in water (Fig. 2) because this solvent cannot penetrate the star interior.

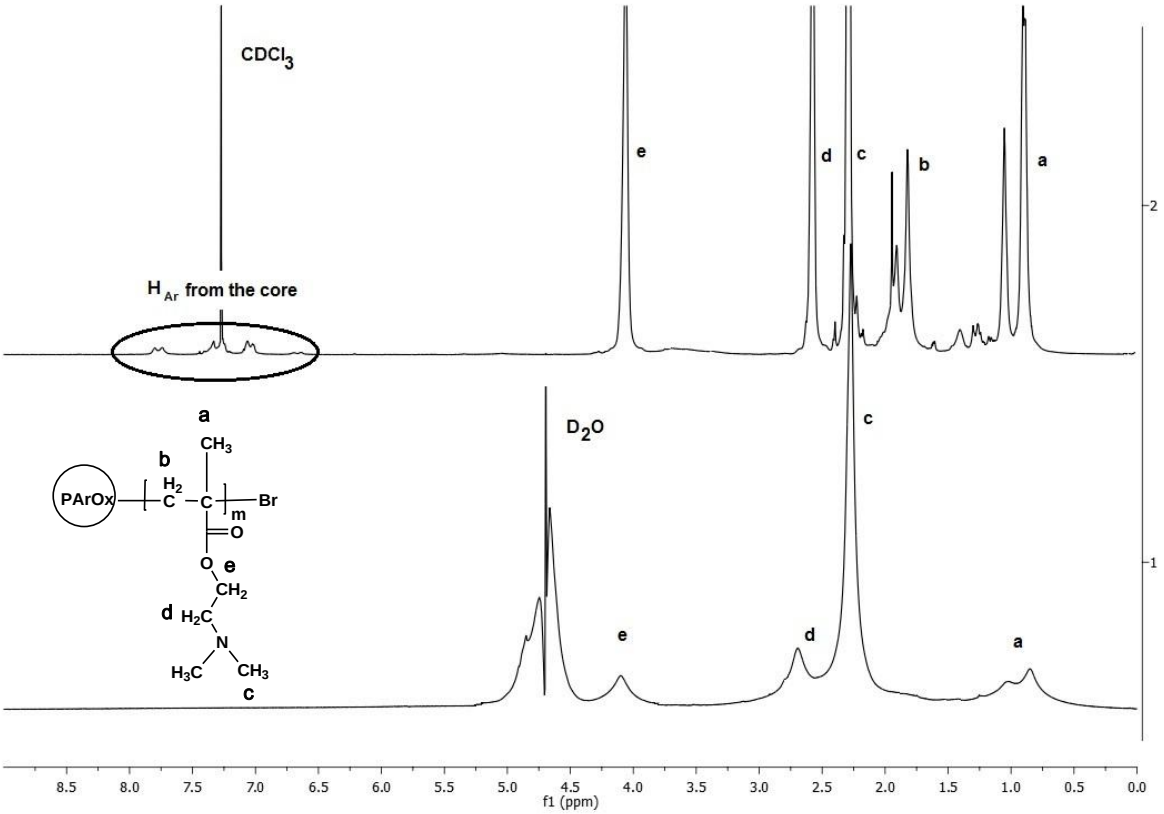


Fig. 2. ^{1}H-NMR spectra of the S7 star in $CDCl_3$ and D_2O (600 MHz).

3.2. Solution properties of the star polymers with PDMAEMA arms

Dynamic light scattering was used to measure the hydrodynamic radii of the PDMAEMA stars in acetone, ethanol, water and DMEM culture media. The measurements were performed at concentrations ensuring strong light scattering. The values of hydrodynamic radii $R_h^{90°}$ obtained for PDMAEMA stars, averaged from at least three measurements, are summarized in Table 3.

Acetone was chosen as a good solvent for the PDMAEMA arms and the PArOx core. Water and ethanol were used as selective solvents that were good for the arms, as the core is insoluble in those solvents. Measurements were also performed in DMEM culture medium, which was used in all the transfection experiments. DMEM contains inorganic salts, amino acids, vitamins, glucose and, in a 5% CO_2 environment, possesses neutral pH. The viscosity (0.08228 cP) and refractive index (1.4) of DMEM at 25 °C was taken into account for the calculations in the DLS measurements [43].

In the pure solvents, as well as in DMEM, the size distributions of star polymers showed one rather broad peak (dispersity of the particles sizes $\frac{\mu_2}{\overline{\Gamma}^2}$ from 0.2-0.3).

Table 3. Hydrodynamic radii of the PDMAEMA stars in different solvents.

Sample	$M_{n\ GPC\text{-}MALLS}$ [g/mol]	DP arm	Contour length[a] [nm]	$R_h^{90°}$[nm] Acetone[b]	$R_h^{90°}$[nm] Water[c]		$R_h^{90°}$[nm] Ethanol[d]	$R_h^{90°}$[nm] DMEM[e]
S2	100 000	18	4.5	7.0	15.0[f]	21.8[g]	14.5	12.5
S7	370 000	79	19.9	9.4	19.0[f]	24.0[g]	15.0	19.5
S8	450 000	98	24.7	14.5	22.5[f]	29.0[g]	21.2	20.1

[a] calculated for fully stretched arms
[b] c = 10 mg/mL
[c-e] c = 1 mg/mL
[f] pH of star dissolution in water
[g] pH=2 for stars S2, S8 and pH=3.8 for star S7

The lowest sizes of the stars were measured in acetone – a good solvent for the star elements. This result indicates that, in acetone solutions, the stars existed as single macromolecules.

In selective solvents – ethanol and water – the obtained sizes were similar but were higher than in acetone, suggesting that the macromolecules were aggregated. Analogous behavior was observed by the Patrickios group [7, 8, 44] for stars with varying functionality (*f* from 7 to 72) and arm lengths (DP from 10 to 100) made of a DMAEMA homopolymer as well as DMAEMA-*b*-[hexa(ethylene glycol) methacrylate] copolymers. After direct dissolution in water, these stars tend to aggregate.

The contour length of arms, calculated by multiplying the DP of arm times 0.252 nm (the contribution of one monomer unit), was compared with the values of R_h measured by DLS in water of acidic pH. At low pH values the electrostatic repulsion between protonated tertiary amine groups results in stretching of arm chains. For all star polymers the measured values of Rh are higher than the contour lengths, in the case of the star with the lowest DP of arm even four times higher, what proves that in water we observe the aggregated structures.

The solution behavior is a significant feature for determining the utility of the amphiphilic, stimuli sensitive stars for biological use. A large number of biological experiments, including cell viability and transfection experiments, have to be performed in culture media. Therefore, the solution behavior of pure stars in DMEM medium was also evaluated. In DMEM, the sizes of stars S2, S7 and S8 were similar to those observed in the selective solvents (water and

ethanol), indicating small aggregates of macromolecules, but were still in the range of dozens nanometers, which is desirable for biological applicability of these polymers.

pH sensitivity of stars in water

To discuss the results of the biological tests, the fact that the PDMAEMA stars are pH sensitive in water [22, 24, 45] should be taken into consideration. At acidic pH, the tertiary amino groups in the arms are completely charged.

Electrostatic repulsion between protonated arms results in stretching and increases the star sizes. At basic pH, the PDMAEMA chains are deprotonated, causing the chains to collapse and decreasing the sizes of the stars.

The effect of pH on the sizes of the stars (samples S2, S7 and S8, Table 1) was studied by dynamic light scattering. The star polymers were directly dissolved in deionized water. The pH of the solutions was adjusted by adding appropriate amounts of 1 M KOH or 1 M HCl.

The pH-responsive behavior resulted in the strong changes in star sizes (Fig. 3). The conformational change of the DMAEMA star size is connected with the change in the degree of ionization of arms. The effective pK values of the ionizable amine groups in DMAEMA star polymers, may be found in the literature, to be around 7 [7, 8]. No abrupt changes of star sizes is seen in this region of pH. With increasing pH, the size of stars decreases by approximately two times. The shrinkage observed is caused by reduced electrostatic repulsion between star arms, resulting in the collapse of the star shell.

An analogous dependence on pH was reported by Zhong et al. [46] for stars with block copolymer arms of ε-caprolactone and DMAEMA linked to a hyperbranched polyester core, which exhibited a twofold decrease in the hydrodynamic diameter when the pH was changed from 2 to 11.

When the pH was increased above 13.5, the polymers precipitated from the solution. Similar limited solubility of star polymers at high pH, caused by the precipitation of collapsed uncharged stars, has been reported by other groups. Dong et al. [22] described similar pH dependent behavior of PDMAEMA stars with silica cores. A decrease in the size of the nanoparticles took place in the range of pH from 6 to 8, while at pH values higher than 8 precipitation was observed. The authors explained this phenomenon by the appearance of strong hydrophobic forces resulting in the flocculation of particles at high pH. In our previous

work, pH sensitive polyacidic stars with a PArOx core exhibited strong phase separation at acidic pH values [41].

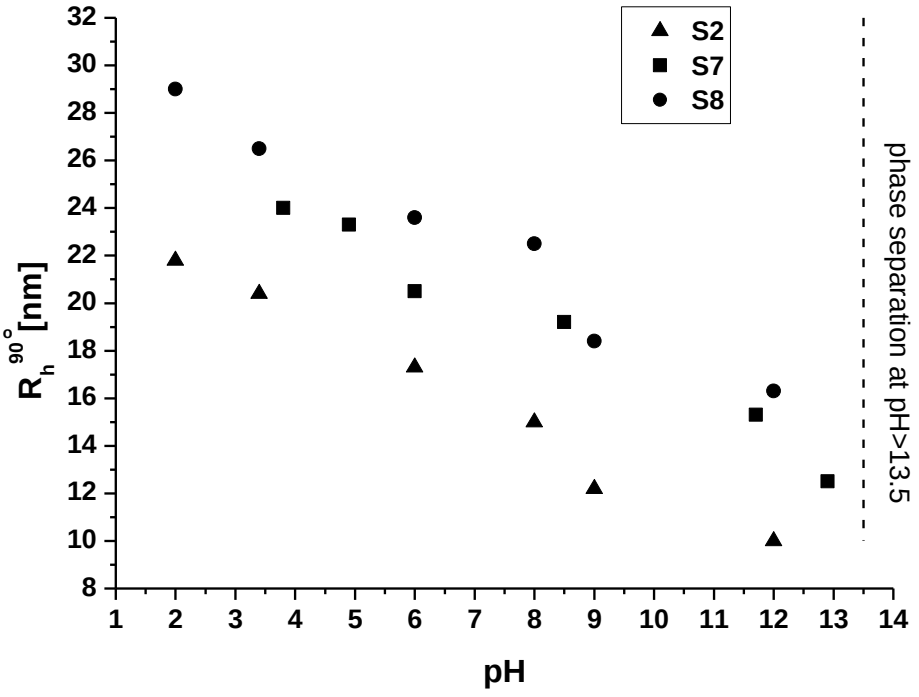


Fig. 3. pH sensitivity of S2, S7 and S8 stars in aqueous solution (c=1 mg/mL).

The distribution of the hydrodynamic radius of the star S7 at different pH values is presented in Fig. 4. A monomodal size distribution that narrowed with increasing pH was observed. The dispersity of the particles sizes ($\frac{\mu_2}{\overline{\Gamma}^2}$) changed from 0.27 at pH=3.8 to 0.107 at pH=11.8, reflecting a more uniform, compact structure of the stars. This result was likely caused by the loss of positive charges at higher pH, and thus reduced electrostatic repulsion.

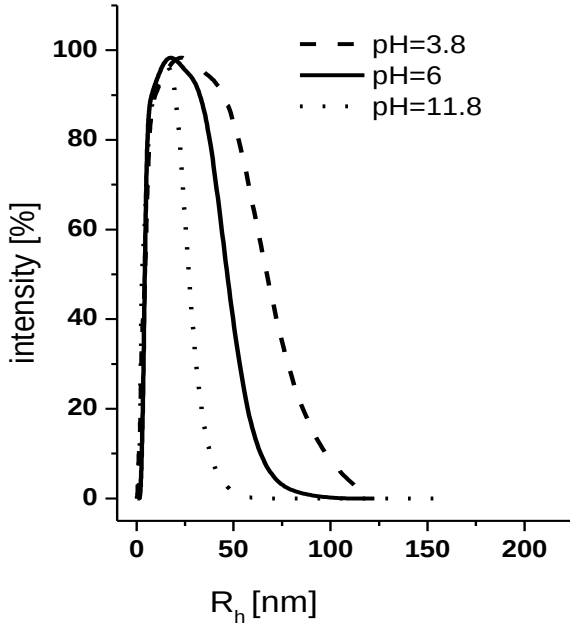


Fig. 4. Distribution of the hydrodynamic radius (intensity weighted distribution) of star S7 at c=1 mg/mL in water at different pH at 25 °C and θ=90°

Thermoresponsivity of stars

Poly(N,N-dimethylaminoethyl methacrylate) is a very well know thermoresponsive polymer that exhibits a pH and molar mass dependent cloud point temperature [45, 47].

The responses of the obtained stars to temperature were investigated using UV-Vis spectroscopy. The T_{cp} values were determined as the temperatures at which the transmittance of the polymer solution decreased to 50% (Table 4).

After dissolution in pure water, the pH of the solutions of the stars was alkaline (pH between 8 and 9), and in these conditions the solutions did not exhibit a cloud point temperature. In addition, T_{cp} was not observed while heating the samples to 90 °C in the pH range from 2 to 12. When the pH was equal 13, the solutions of the stars turned to a white opaque suspension as the temperature was increased. The effect of increasing solution temperature at different pH on PDMAEMA stars with different number and length of arms was studied in details by Tsukruk et. al. [48].

Below pH 12, the lack of thermoresponsivity of the stars can be attributed to the repulsive electrostatic interactions between partially protonated chains, which are strong enough to prevent their collapse upon a change of temperature. Additionally, in DMEM culture media with a neutral pH, the phase transition was not observed.

At pH>13 the arms are deprotonated. As the temperature is increased, hydrophobic interactions dominate and the polymer become insoluble. Similar behavior was observed for C_{60}-fullerene PDMAEMA structures [21]. In that study, the authors observed that the solutions lost their temperature responsiveness in acidic conditions, while this behavior was recovered in alkaline conditions.

UV-Vis measurements of the transmittance of the star solutions showed that the T_{cp} of the stars strongly depended on their molar mass. The values for the phase transition temperature decreased with increasing PDMAEMA arm lengths (Fig. 5) at pH 13.0.

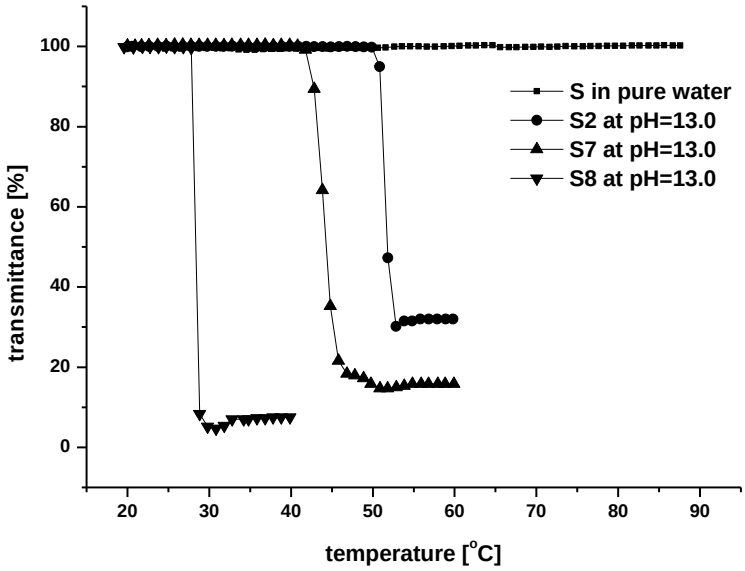


Fig. 5. Transmittance of stars in an aqueous solution at λ=700 nm as a function of temperature.

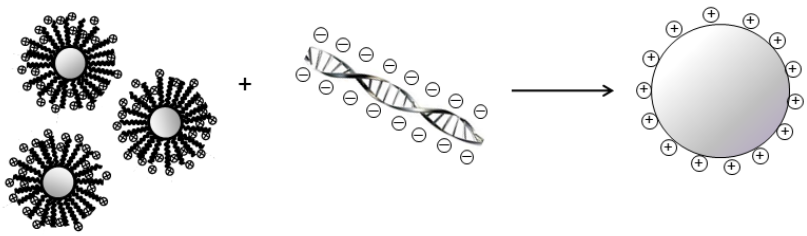
The results obtained for the stars studied in this work are consistent with results of the Bao group concerning stars with a hyperbranched alanine based poly(ester amide) core and PDMAEMA arms of different lengths [31]. In their study, increasing the DP of the arms from 5 to 71 caused a decrease of T_{cp} from 67 °C to 35 °C in PBS buffer at pH=8.0.

The opposite trend was observed by Patrickios [44], who reported increased values of T_{cp} for 19 arm PDMAEMA stars at pH=8.5 (dissolution pH) with increasing arm lengths. Increasing the DP of the arms from 11 to 84 led to an increase in T_{cp} from 43 °C to 56 °C . The authors explained this increase by better steric stabilization of the star in aqueous solution with longer arms of PDMAEMA.

3.3. Complexation of star polymers with DNA

It is known that polybases bearing positive charges bind negatively charged DNA into particles called polyplexes (Scheme 2), which are small enough to enter into cells via the endosomal pathway [4, 33]. In this study, we used S2, S7 and S8 stars to complex plasmid DNA and characterized the efficiency of these polyplexes in transfection experiments.

The polyplexes were formed at different N/P ratios in DMEM culture media using the same conditions as used for transfection experiments. A 4784 base pair plasmid DNA coding luciferase (pMetLuc2-Control) was used. An appropriate amount of star solution in DMEM was added to a fixed amount of DNA, which is more efficient than adding in the reverse order [43]. The solutions were incubated for 30 minutes at room temperature. Polyplex formation was investigated by DLS and by agarose gel electrophoresis.



Scheme 2. Complexation of star polymers with DNA into a polyplex.

Agarose gel electrophoresis is a simple method to evaluate the formation of polyplexes. The appropriate ratio of N/P at which the nucleic acid was fully bound to the polycation star was estimated. Naked pDNA was used as a control and is shown in the first lane at N/P=0 in Fig. 6. The amount of star polymer needed for total binding of the nucleic acid decreased with increasing DP of the star arms. Complete condensation of the DNA was observed at N/P ratios 6, 4 and 3 for the stars S2, S7 and S8, respectively. Neoh et al. [16] observed a similar dependence for star polymers with β-cyclodextrin core and PDMAEMA arms of DP from 16 to 99.

The N/P values required for complete condensation for the studied stars were higher than for branched PEI, where complete condensation of DNA was obtained at N/P=2.

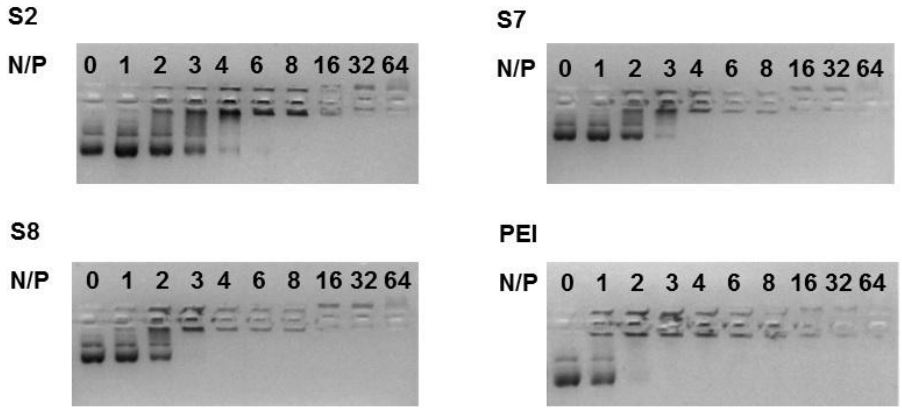


Fig. 6. Electrophoretic mobility of plasmid DNA in the presence of star polymers and branched PEI at different N/P ratios.

The sizes of the polyplexes (S2-pDNA, S7-pDNA, S8-pDNA) at different N/P ratios were determined by DLS. These results are shown in Fig. 7.

The sizes of the star polymer-pDNA complexes are shown in Fig. 7. All $R_h^{90^\circ}$ sizes for the polyplexes were higher than that of pure pDNA, which had a $R_h^{90^\circ}$ of approximately 100 nm, with monomodal but broad distribution $\frac{\mu_2}{\overline{\Gamma}^2}$ =0.3.

The hydrodynamic radii of the polyplexes formed with stars S2 and S7 decreased with increasing N/P ratio over the studied range (Fig. 7). Increasing N/P from 1 to 16 caused a change in the $R_h^{90^\circ}$ of approximately 70 nm. A similar trend was observed by Mueller et al. [49] for PDMAEMA 5-arm stars complexed with plasmid DNA encoding green fluorescent protein. The $R_h^{90^\circ}$ of their star polyplex decreased by 10 nm with a change in N/P from 1 to 12. Neoh et al. [16] reported a significant decrease in sizes from 300 nm to 90 nm over the same range of N/P ratios for polyplexes of PDMAEMA stars with 4 arms complexed with plasmid DNA encoding *Renilla* luciferase.

The size of polyplexes made of star S8 (S8-pDNA) increased to a maximum of 400 nm at N/P=4, and then decreased at N/P=6. Similar behavior was observed by Tam et al. [20] for polyplexes made of 4-arm stars with poly(ethylene oxide)-*b*-poly(2-(diethylamino)ethyl methacrylate) (PEO-*b*-PDEAEMA) arms complexed with DNA (10 kilobase pair). The authors explained the size enlargement by rearrangement of the DNA structure. Mueller et al. [24] observed an increase in size for nanoparticles with a magnetic core, silica shell and PDMAEMA corona complexed with plasmid DNA pEGFP-N1 (4.7 kilobase pair). They assumed bridging of two hybrid nanoparticles with one pDNA.

Above N/P=4, when pDNA is completely bound by star S8, the sizes of the polyplexes decreased, as for other stars.

For all obtained polyplexes at N/P= 8, the $R_h^{90°}$ values were close (approximately 150 nm), and there were no significant changes in the sizes when N/P was increased to 16. This polyplex behavior is consistent with that reported in the literature [16, 20]. Polyplexes formed more compact structures than the naked plasmid DNA, and the dispersity of their sizes $\frac{\mu_2}{\overline{\Gamma}^2}$ did not exceed 0.15, which was much lower than for pDNA.

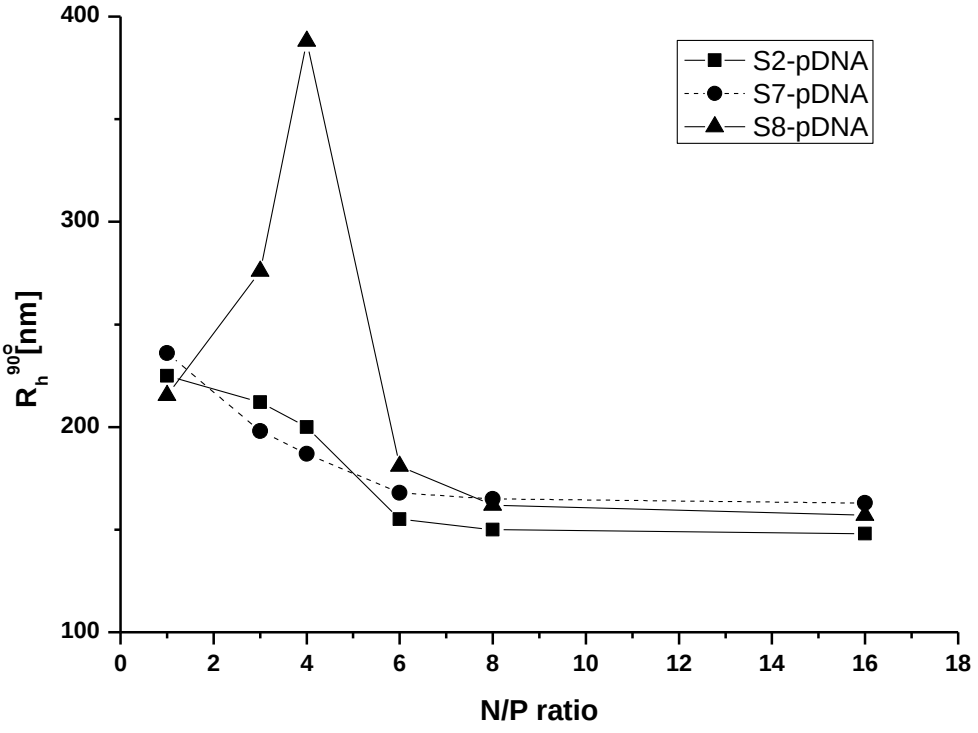


Fig. 7. Average hydrodynamic radii of polyplexes at different N/P in DMEM (the lines are guides for the eye).

Positively charged particles may interact with negatively charged cell surfaces, which enables their cellular uptake. Thus, the surface charge of polyplexes is an important property for evaluating a polyplex formulation in transfection studies. It is known that positive zeta potential is needed for successful polyplex endocytosis [32].

In order to determine the surface charges, the zeta potentials of the star polymers in aqueous solution and in DMEM culture medium and of star polyplexes in DMEM were measured at different N/P ratios. In DMEM, the zeta potential was lower than in water. However, in both cases it was positive (Table 4).

Table 4. Zeta potential of the PDMAEMA stars.

Sample	Zeta potential in aqueous solution at pH=7.4 [mV]	Zeta potential in DMEM at pH=7.4 [mV]
S2	30.0	15.8
S7	50.0	18.6
S8	56.3	20.0

For star polyplexes, the zeta potential values versus the N/P ratio are plotted in Fig. 8. For all the star polyplexes at N/P≥4, the zeta potential was positive and varied between 10 and 20 mV. Above N/P=6, the excess of star used for polyplex formation did not influence the zeta potential.

Stars S2 and S7 required higher N/P ratios compared to S8 to reverse the surface charge. For preparation of the star polyplex S8-pDNA with positive zeta potential, a lower amount of star was needed in comparison with those described above (N/P=3).

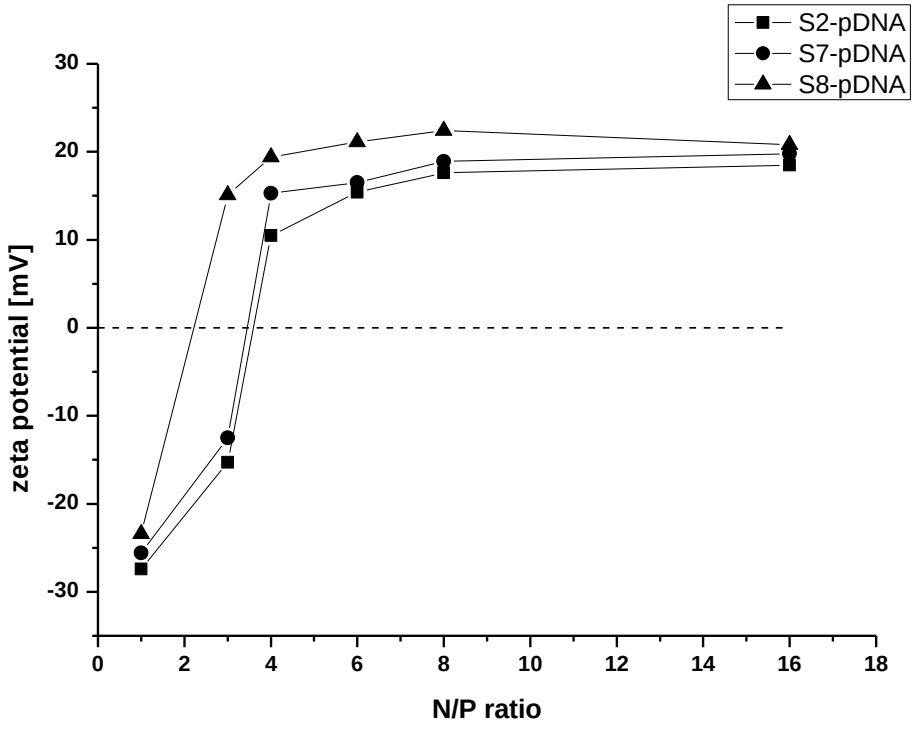


Fig. 8. Zeta potential of the star polyplexes at different N/P ratios in DMEM at pH=7.4 (the lines are guides for the eye).

The structures of the stars, pDNA and polyplexes were characterized using cryo-TEM. The star particles in the TEM micrograph shown in Fig. 9 have a globular structure. The sizes of

the stars are summarized in Table 5. The values obtained were smaller than those measured for the star macromolecules by dynamic light scattering in aqueous solution. It is difficult to find a well-founded reason for this difference. The high dilution may be a possible reason, next to TEM specific factors: the average obtained from TEM is the number average which is generally smaller or corona of the particles might not be fully visible because is highly swollen with solvent.

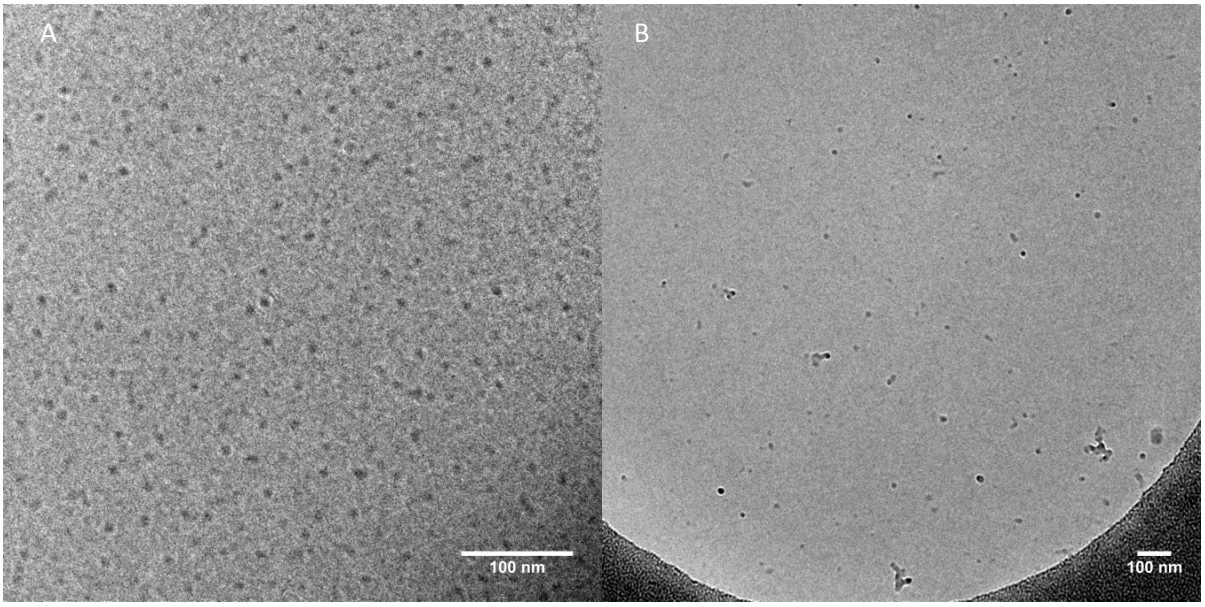


Fig. 9. Cryogenic transmission electron microscopy images obtained from an aqueous solution of stars at a concentration of 1 mg/mL. A) S2 star, pixel size 0.43 nm. B) S8 star, pixel size 0.13 nm.

Plasmid DNA in culture media DMEM appeared either as a hairy aggregate (Fig. 10 A) or as a single, tangled particle with a longitudinal size of 600 to 800 nm (Fig. 10 B). These results are similar to those reported by Rudolph et al. [50], who showed that plasmid DNA encoding luciferase in HBS solution was observed as tangled particles by TEM.

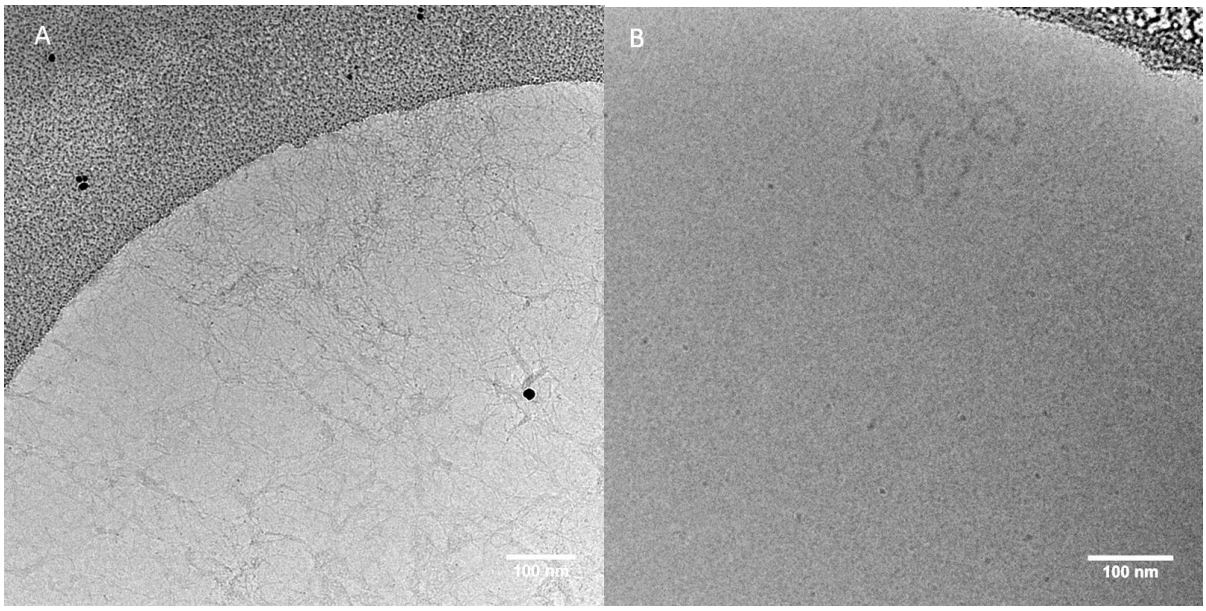


Fig. 10. Cryogenic transmission electron microscopy images obtained from a DMEM solution of pDNA at a concentration of 0.5 mg/mL. A) hairy aggregates, pixel size 0.21 nm. B) single tangled particle, pixel size 0,17 nm.

In the case of polyplexes made of stars S2 and S7 with shorter arm lengths, combined in elongated clusters were often observed in the cryo-TEM pictures (Fig. 11 A, B). Polyplexes obtained from the star with the longest DP of the arms (8-pDNA) appeared as regular spherical shaped particles by TEM (Fig. 11 C). In this case, the star polymers bind DNA and incorporate its fibers inside the globular structure of the formed polyplex.

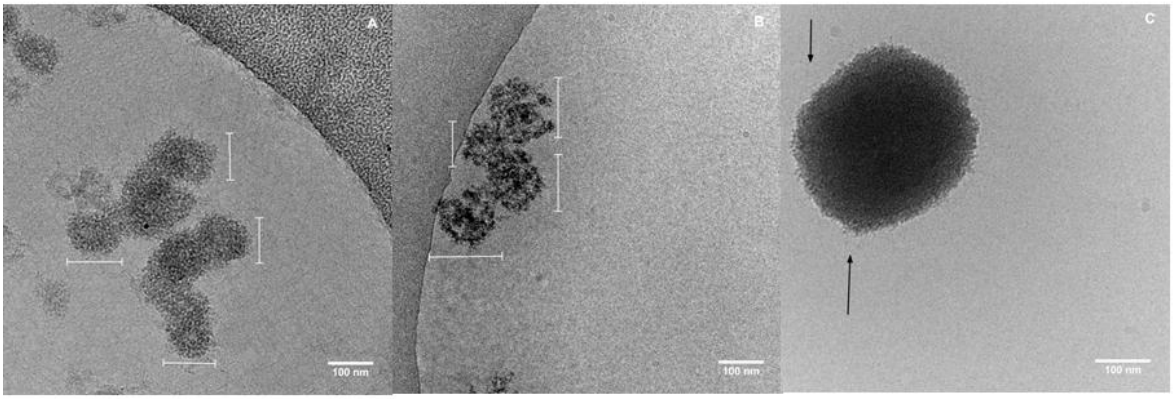


Fig. 11. Cryogenic transmission electron microscopy images obtained from DMEM culture media solution of star-pDNA at N/P=16. A) S2-pDNA, pixel size 0.21 nm. B) S7-pDNA, pixel size 0.21 nm. C) S8-pDNA, pixel size 0.17 nm.

Table 5. Sizes of stars, pDNA and polyplexes measured in the TEM micrographs.

Diameter of star [nm] [a]	**Length of DNA strands [nm]**	**Diameter of polyplexes [nm]** [a]

Star S2	Star S7	Star S8	pDNA	S2-pDNA	S7-pDNA	S8-pDNA
9	12	20	600-800	112	134	356

[a] diameter of particle was calculated as average of size of all objects visible in the picture.

3.4 Cytotoxicity and transfection efficiency studies

Cytotoxicity of polycationic polymers is one of the factors that must be evaluated before the use of these polymers for applications in biological systems. The amine groups of DMAEMA are potentially toxic and disruptive to the biological membranes of cells, and they could cause apoptosis or necrosis. For linear PDMAEMA, it was previously observed that cellular membrane disruption and interactions with negatively charged proteins in the intracellular space strongly increase with increases in the molar mass [51].

Star polymers S2, S7 and S8 were used to examine the effect of DP of the arms on cell viability. HT-1080 fibrosarcoma cells, which were also used to study transfection, were mixed in DMEM culture medium DMEM with the stars. Branched polyethylenimine PEI with a molar mass 25 000 g/mol, a commercially purchased transfection reagent, was used for comparison. The viability of the HT-1080 cells was assessed by AlamarBlue reduction test assay. The results plotted in Fig. 12 show that cells viability was significantly lowered as the length of the star arms was increased. As seen by comparing the results in Fig. 12, the PDMAEMA stars were considerably less toxic than branched PEI. No living cells were detected at PEI concentrations higher than 50 μg/mL. A similar trend was reported for other DMAEMA star polymers and different cell types [9, 16].

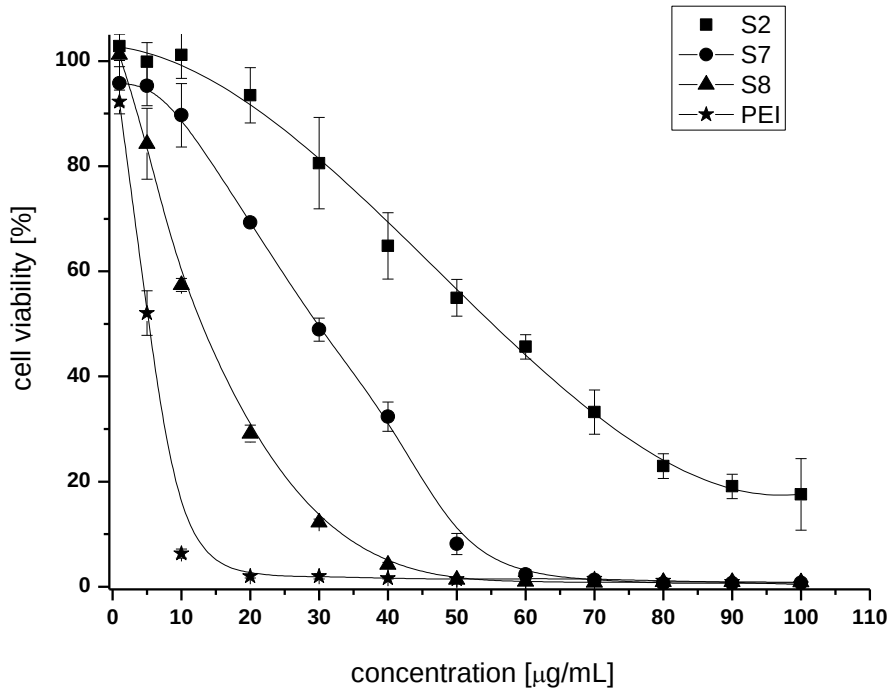


Fig. 12. Effect of S2, S7, S8 stars and branched PEI concentrations on HT-1080 cell viability (the lines through data points are guides for the eye).

Transfection efficiency is the sum of several factors, including the stability of the polyplexes, their cell uptake, the endosomal escape of the DNA and its rate of entering the nucleus. The method used to assay transfection efficiency here was based on estimating the overall protein production by transfected cells. It was assumed that only living cells manufactured proteins at the time point of measurement. The transfection efficiency, visible as luciferase activity, was evaluated indirectly as an average of three luminescence measurements. However, the obtained results had to be analyzed together with the cytotoxicity of polyplexes. The in vitro gene transfection efficiency of HT-1080 cells, mediated by cationic star-pDNA complexes, has been tested using secreted Metrida luciferase as a reporter gene driven by the CMV promoter. In contrast to other commonly used luciferases, this protein is secreted from the cells directly into the culture medium, where its activity can be measured. Therefore, there is no need for cell lysis, and the measurements are less prone to errors. This assay was performed using a constant amount of plasmid DNA complexed with stars S2, S7 and S8 at different N/P ratios. The same panel for branched PEI served as a positive control. The results of the transfection experiments are summarized in Fig. 13. The cytotoxicity of the tested transfection conditions was simultaneously assayed, and the results are plotted in Fig. 14.

Luciferase expression mediated by the tested polymers was dependent on the N/P ratio. For polyplexes of the studied stars, the luciferase activity significantly increased at N/P=8 for S7-

pDNA and S8-pDNA and at N/P=16 for S2-pDNA. At these N/P values, all the polyplexes had positive zeta potentials, which is relevant for interaction of the polyplex with the cell membrane and enabling uptake by the cells [52].

The maximum transfection efficiency was obtained at ratios of 64, 32 and 16 for polyplexes made of stars S2, S7 and S8, respectively. The results for branched PEI indicated the highest luciferase activity at ratios of 6 and 8, followed by down regulation at higher N/P ratios, which corresponded well with data in the literature [53].

The sizes of the polyplexes, their zeta-potential and cytotoxicity should all be taken into account when choosing star polymers best suited for nucleic acid transport. In our case, the polyplex S8-pDNA with stable size ($R_h^{90°}$=160 nm) revealed the best transfection efficiency of all the studied polyplexes at N/P=16 and simultaneously preserved 100% cell viability. The pDNA binding efficiency of star S8 provided the best protection for the nucleic acid, which resulted in the highest transfection efficiency.

As indicated in Fig. 7, at N/P>6 the polyplexes were most compact and their sizes were stabilized. The relevant amounts of stars used at N/P>6 interacted with the nucleic acid, causing its sufficient protection and resulting in a higher efficiency of transfection.

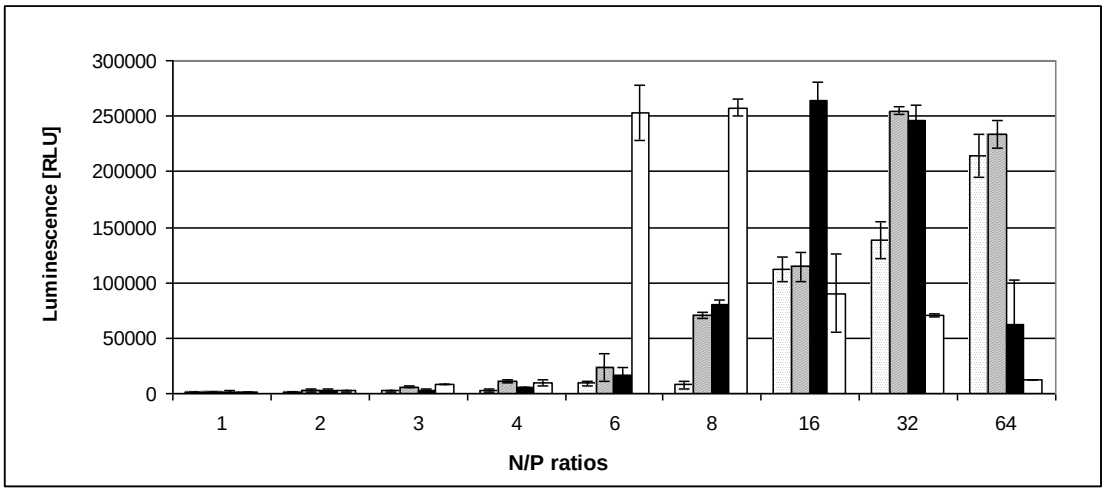


Fig. 13. Efficiency of transfection of HT-1080 with pDNA mediated by polyplexes at different N/P ratios. The results are shown as overall luminescence values. (S2-pDNA – dotted, S7-pDNA – dashed, S8-pDNA – filed, and PEI-pDNA – open boxes).

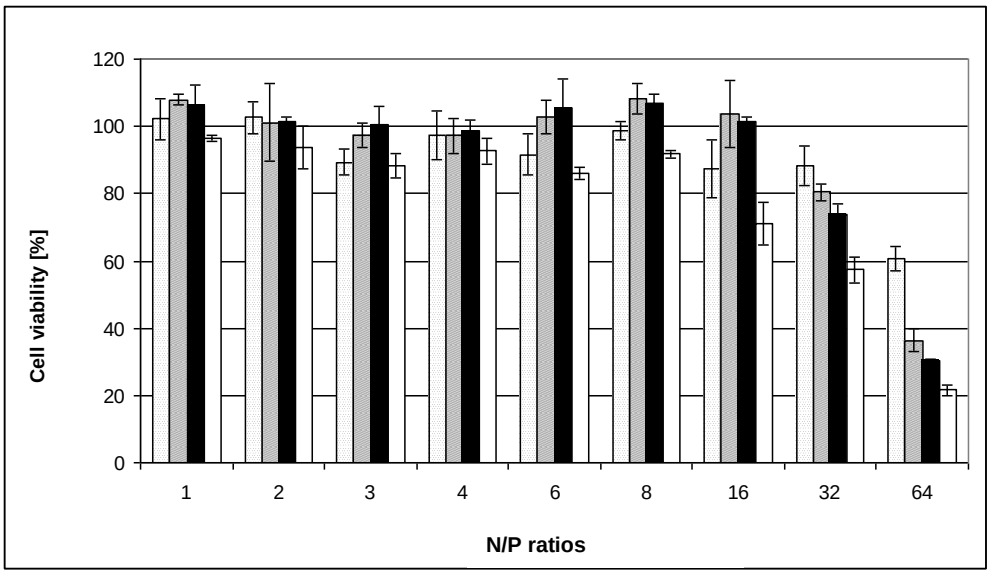


Fig. 14. Toxicity of polyplexes for the transfection conditions. The results are shown as a percentage of the control, where untreated cells constituted 100%**.** (S2-pDNA – dotted, S7-pDNA – dashed, S8-pDNA – filed, and PEI-pDNA – open boxes).

4. CONCLUSIONS

A series of 28-arm DMAEMA star polymers with a hyperbranched poly(arylene oxindole) core were synthesized in a controlled manner using ATRP. The DP of the arms and polymer/solvent interactions were found to influence the sizes of the stars in solution. In a good solvent (acetone), the $R_h^{90^\circ}$ values indicated that the stars existed in solution as isolated macromolecules. In a selective solvent (water), higher values of $R_h^{90^\circ}$ were obtained, which indicated that small aggregates were present in water. The stars were pH-sensitive, and their sizes decreased by two times with an increase of pH from 2 to 13 because of shrinkage of their unprotonated arms at high pH. They also exhibited thermoresponsiveness in aqueous solution at high pH. The cloud point temperature decreased significantly with increases in the DP of the star arms from 18 to 98. The synthesized stars were able to bind plasmid DNA to polyplex nanoparticles with $R_h^{90^\circ}$ values equal to 100-200 nm and positive zeta potentials of 10-20 mV, which are desirable for interaction with negatively charged cell membranes. The high transfection efficiency achieved with these polyplexes, tested using HT-1080 cells, showed that the studied systems are prospective gene delivery agents.

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